STUDIES ON THE FERTILIZIN-GEL DISPERSING ENZYME IN EXTRACTS OF *MELLITA QUINQUIESPERFORATA* SPERMATOZOA

By

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INTRODUCTION

The nature of the chemical substances involved in the sperm-egg interactions leading to the activation of the eggs has been of great interest to embryologists for many years. Lillie (1912) demonstrated that the supernatant of a suspension of eggs from the annelid, Nereis limbata, and from the sea urchin, Arbacia punctulata, cause agglutination of homologous spermatozoa. Lillie applied the term "fertilizin" to the agent responsible for the agglutination. Frank (1939) showed that extracts of the spermatozoa of A. punctulata contained a substance which neutralized the agglutinating capacity of fertilizin and caused the agglutination of homologous eggs. Tyler (1940a) demonstrated the presence of a similar substance in the spermatozoa of the key-hole limpet, Megathura crenulata. Tyler applied the term "antifertilizin" to the substance extracted from the spermatozoa. Lillie (1914) had suggested that the cytoplasm of the eggs of some species possessed a substance which neutralized the effect of fertilizin, and Tyler (1940b) succeeded in extracting such a substance from sea urchin eggs which he found to be similar to the antifertilizin from spermatozoa.

Lillie (1912) proposed that fertilizin functioned in the specific attachment of the spermatozoa to the egg. The discovery of antifertilizin gave impetus to this hypothesis. Although there are those that

hold contrary views (see Hagström, 1956) It is generally held that the fertilizin on the surface of the egg combines with the antifertilizin of the spermatozoa in a manner analogous to antigen-antibody reactions, resulting in the attachment of the spermatozoa to the egg. Lillie (1919) demonstrated that fertilizin was not obtained from tissues other than eggs. This implicated fertilizin as the agent responsible for the tissue-specificity of fertilization. The species-specificity of fertilization has also been demonstrated, based on the fertilizin-antifertilizin reaction (see review by Tyler, 1948). The reaction between fertilizin and antifertilizin of heterologous species may occur, if the species are closely related, but sperm agglutination titer in no way approaches the agglutination apparent when homologous materials are used. In most echinoids, the agglutination of spermatozoa by fertilizin is of a transient nature, the agglutination reversing after a short period. The reversed spermatozoa are incapable of re-agglutination and are generally incapable of fertilizing eggs. This indicates that the antifertilizin has been neutralized by the fertilizin. Tyler (1955) attributes the reversal of agglutinated spermatozoa to the splitting of the individual fertilizin molecules that bind the spermatozoa together.

The eggs of many species of animals are surrounded by membranes, follicle cells and/or gelatinous material through which the spermatozoa must pass before fertilization can occur. In echinoids, in addition to the membranes of the egg surface, the egg is surrounded by a layer of gelatinous material that has been shown to be identical with fertilizin (Tyler and Fox, 1940; Tyler, 1941). A considerable amount

of work has been done to elucidate the chemical nature of the fertilizin-gel. Tyler (1949a) has shown fertilizin to be a glyco-protein. It is acidic, with a high sulphate content (ca. 25 per cent). Vasseur and Immers (1949) have shown that the sugar component of fertilizin varies, depending upon the species from which the fertilizin is obtained. The latter workers demonstrated that the sugar was galactose in Echinus esculentus, fucose in Echinocardium cordatum, fucose and galactose in Strongylocentrotus drobachiensis, and fucose and glucose in Paracentrotus lividus. These workers suggested a genus-specificity of fertilizin, based on the sugar component; however, other workers consider it premature to assess a genus-specificity on this basis (see Tyler, 1955).

It has been demonstrated that the spermatozoa of many species of animals possess a substance which is capable of breaking down the membranes or dispersing the follicle cells or fertilizin-gel surrounding the eggs of homologous species. Such substances have been reported as present in amphibians (Hibbard, 1928; Wintrebert, 1929), gastropods (Tyler, 1939; von Medem, 1942), mammals (Yamane, 1930; Pincus, 1930; Duran-Reynals, 1942), pelycepods (Berg, 1949) and polychaetes (Osterhout, 1950; Colwin and Colwin, 1958). The most widely known of these lytic or dispersing agents is hyaluronidase, obtained from mammalian testes. The presence of similar substances in echinoids has been the source of much discussion since Hartmann, et al. (1940) reported that a gel-dispersing agent could be obtained from sea urchin spermatozoa by precipitation of the seminal fluid with methanol. Tyler and O'Melveny (1941) proposed that this apparent dispersal was due to the activity of antifertilizin and postulated that no substance was then demonstrable

in sea urchin spermatozoa which would depolymerize the fertilizin-gel. Runnstrom, et al. (1942) produced a methanol extract of the spermatozoa of Psammechinus miliaris and Echinocardium cordatum that liquefied the vitelline membrane of homologous unfertilized eggs. Monroy and Ruffo (1947) extracted the spermatozoa of Arbacia lixula and Sphaerechinus granularis with 0.1 N acetic acid and reported a substance from this extraction which, they claimed, caused the removal of the fertilizin-gel of homologous eggs. Krauss (1950), using methods employed in the extraction of hyaluronidase from mammalian testes, was unable to find a gel-dispersing agent in the spermatozoa of Strongylocentrotus purpuratus and Lytechinus pictus. Vasseur (1951) made a solution from the fertilizin-gel surrounding the eggs of Paracentrotus lividus and found that live sperm caused a decrease in the viscosity of this solution. From these results Vasseur concluded that the spermatozoa possessed an enzyme capable of depolymerizing the fertilizin-gel. Vasseur's conclusion was questioned by Monroy and Tosi (1952) and by Monroy, et al. (1954). The latter investigators also refuted the earlier work of Monroy and Ruffo (1947). Ishida (1954) reported a gel-dissolving substance in the spermatozoa of Hemicentrotus pulcherrimus and showed that if the spermatozoa were first rendered nonfertilizable by treatment with fertilizin, the gel-dissolving substance was still active. Metz (1957) and Brookbank (1958) pointed out that when live spermatozoa were used, there remained the possibility that acid conditions, caused by the production of carbon dioxide by the spermatozoa, could cause the dissolution of the fertilizin-gel. In this report, Brookbank demonstrated the presence of a gel-dispersing agent in the

spermatozoa and sperm extracts of the sand dollar, Mellita quinquesperforata, that was separable from antifertilizin on the basis of temperature and pH sensitivity. In a short note, Hathaway, et al. (1960) reported that sodium-lauryl-sulphate-extracts of A. punctulata spermatozoa caused the removal of the fertilizin-gel from homologous eggs if antifertilizin was first removed from the extracts by precipitation and centrifugation.

From the reports cited, it is evident that the spermatozoa of at least some species of echinoids possess lytic agents or depolymerases which are active against the fertilizin-gel surrounding the eggs of homologous species. There is also good evidence that the agent responsible for the lytic or depolymerase activity is not antifertilizin. The accumulation of evidence in support of the presence of these agents has made it desirable that more information be secured concerning the nature of the agents. Tyler (1939, 1948) reported that the agent responsible for the lysis of the vitelline membranes of Megathura crenulata eggs had properties of proteins. The substance extracted by Runnstrom, et al. (1942) was dialyzable and believed to be an 18-carbon fatty acid. Brookbank (1958) suggested that the gel-dispersing agent from M. quinquesperforata spermatozoa was a protein and possible an enzyme.

The present dissertation describes studies undertaken to further elucidate some of the characteristics of the gel-dispersing agent from the spermatozoa of the sand dollar, M. quinquesperforata and to further examine the hypothesis that the gel-dispersing agent is distinct from antifertilizin.

MATERIALS AND METHODS

Sand dollars were collected from three localities. In the localities where collections were made the habitats are exposed at low tide levels. When possible, collections were made when the animals were thus exposed. At other times, collections were made by dredging. Most of the animals were collected from sand flats on the northeastern side of Sea Horse Key, which lies approximately three miles west of Cedar Key, Florida. In July, 1960, animals were collected from Bogue Sound near the Duke University Marine Laboratory, Beaufort, North Carolina. The animals were not fully "ripe" at this time. In January, 1961, approximately 200 animals were collected from sand flats on the southern extremity of La Costa Island, Florida. Gametes may be secured in large quantities from the sand dollar, M. quinquiesperforata, during the months of April and May and during the period October-December in the area adjacent to Sea Horse Key. In the Beaufort area the spawning seasons are, apparently, slightly later in the spring and earlier in the fall. Temperature appears to be a major factor determining the extent of the spawning season. Water temperatures range from 20°C. to 26°C. during the spawning season. When low tide levels occur at night the animals may be exposed to lower temperatures, especially in the fall months. Exposure to low temperatures generally signals the end of the spawning season. As water temperatures rise during the late spring there is a gradual decrease in the production of gametes.

During the course of the work at the Duke University Marine Laboratory, animals were kept in running sea water until needed. From the other localities, animals were transported, without water, to the University of Florida and maintained in aerated sea water at 12° C. Under these conditions, the animals were suitable for use for about ten days.

Gametes were secured by forced spawning, induced by injecting approximately 5 milliliters of 0.5 N potassium chloride into the perivisceral cavity (Palmer, 1937; Tyler, 1949b). Gametes were collected in Syracuse dishes filled with filtered sea water.

Preparation of sperm extracts

Essentially "dry" sperm were centrifuged for 30 minutes at 2000 rpm in an International Model CM centrifuge. The supernatant was decanted and three volumes of filtered sea water were added to the sedimented spermatozoa. The spermatozoa were then thoroughly mixed with the sea water and this suspension was frozen in 13 x 150 millimeter test tubes for at least 24 hours at -9° C. While frozen, the mixture was homogenized with a motor-driven pestle. The resultant viscous mass was centrifuged for 30 minutes at 15,500 x gravity in an SS -1 Serval centrifuge. The supernatant fluid from this centrifugation was retained as crude extract. The pH of the extract was adjusted to 8.0 and buffered with 0.2 M phosphate buffer. When possible, a sufficient quantity of spermatozoa was used to yield approximately 40 milliliters of crude extract from each preparation. In the later stages of this study, a Serval refrigerated centrifuge with an SS -34 rotor was used. With this equipment all centrifugations were made at 0° C.

As the first step in purification the crude extract was dialyzed against 60 volumes of filtered sea water at 1° C. for 24 hours. During this dialysis, the sea water was changed three time. The dialysate contained no gel-dispersing agent, and was discarded. After dialysis the extract was divided into two portions. One portion was retained as crude extract. Ammonium sulphate was added to the second portion according to the procedure developed by Kunitz (1952) until the solution was 60 per cent ammonium sulphate by volume, at which point a white precipitate formed. Precipitation was allowed to proceed for at least 12 hours at 1° C. The precipitated extract was then centrifuged at 15,500 x gravity for 10 minutes and the supernatant fluid was discarded, after tests revealed no gel-dispersal activity in the supernatant fluid. Cold, filtered sea water was added to the precipitate in a volume equal to the original volume of extract prior to the addition of ammonium sulphate. The re-constituted extract was then dialyzed against 60 volumes of filtered sea water, through three changes of sea water, for 24 hours at 1° C. to remove the ammonium sulphate. The dialysate contained no active gel-dispersing agent and was discarded. After this dialysis the pH of the extract was checked and if necessary adjusted to pH 8.0. In practically all cases, the pH varied less than 0.2 units. Qualitative (barium chloride) tests for residual ammonium sulphate were not performed. Extracts prepared by ammonium sulphate precipitation were regarded as partially purified extracts and henceforth will be referred to as such. All extracts were stored at 1° C. until used. All tests for gel-dispersal activity of the extracts were made at 25° C.

Preparation of eggs

The eggs of the sand dollar, M. quinquiesperforata, are essentially spherical and are surrounded by a layer of fertilizin-gel that is approximately 55 micra in thickness. There is a layer of echinochrome granules imbedded in the fertilizin-gel, approximately 15 micra below the surface of the fertilizin-gel (Figure 1). These granules are dispersed when the fertilizin-gel is depolymerized. The fertilizin-gel is transparent, having about the same refractive index as water. Thus, the fertilizin-gel is not visible unless treated in some manner to outline the material.

To determine the volume of fertilizin-gel surrounding the eggs, the material was first made visible with treatment with Janus Green stain. The diameter of the gel was then measured with the use of ocular micrometer. A total of 204 eggs from several animals were thus measured. By subtracting the volume of material within the ring of echinochrome granules from the total volume of fertilizin-gel, it was possible to determine the volume of gel residing outside the ring of echinochrome granules. By this method, the mean volume of fertilizin-gel outside the granules was found to be 7.0×10^{-4} cubic millimeters per egg. The rate of activity of the gel-dispersing agent was estimated by recording the time required for the dispersal of the fertilizin-gel to the point that the echinochrome granules began to fall to the bottom of the Syracuse dish.

In order that the relative concentration of eggs might be controlled, a standard procedure was followed in all experiments requiring the use of eggs. Eggs were secured by forced spawning, as previously

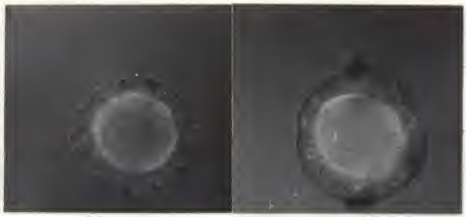

Figure 1. Unfertilized eggs of the sand dollar, Mellita quinquiesperforata. The egg on the right has been stained with Janus Green to outline the fertilizin-gel. X 250.

Figure 2. Egg of the sand dollar, Mellita quinquiesperforata, after exposure to homologous sperm extract. X 250.

described. The eggs were then placed in cold, filtered sea water in a 15 milliliter calibrated centrifuge tube and centrifuged for 1 minute at 1000 rpm. After centrifugation the water was decanted and cold, filtered sea water was added. This procedure was repeated three times with each sample of eggs. After a fourth centrifugation the volume of eggs was noted and after the water was decanted cold, filtered sea water was added until the desired concentration of eggs was reached. This procedure caused no apparent damage to the eggs or to the fertilizin-gel when fresh eggs were used. When needed, eggs were removed from the centrifuge tube with a standard dropping pipette, after the suspension had been gently agitated to insure an even distribution of eggs throughout the suspension. The same dropping pipette was used in all experiments in an attempt to insure a constant number of eggs per drop. Eggs were deemed to be fresh when used within two hours after spawning. A total of 47 drops from 1 per cent egg suspensions (1 ml. of packed eggs and interstitial water plus 99 ml. of sea water) was used to determine the number of eggs per drop. These drops were placed on a grid which had been etched on the bottom of a Petri dish and visual counts of the number of eggs in each drop were made at a magnification of five diameters. By this procedure it was determined that the mean number of eggs per drop in a 1 per cent egg suspension was 87.6, $\pm$ 9.6 eggs per drop. A ten-fold increase in this number, that is, a 10 per cent egg suspension would then contain approximately 876 eggs per drop. On the basis of this calculation and the calculation of the volume of fertilizin-gel per egg, it was determined that the volume of fertilizin-gel outside the ring of echinochrome granules in one drop of a 10 per cent egg suspension was 0.614 cubic millimeters.

Ten per cent egg suspensions were used in all experiments unless otherwise specified.

Determination of rates of gel-dispersal activity at various extract concentrations

To determine the effect of decreased extract concentrations on the rate of removal of the fertilizin-gel by the dispersing agent, two types of experiments were undertaken. In one set of experiments partially purified extract was diluted to various concentrations with filtered sea water. The activity of two drops of the various concentrations was tested against one drop of 10 per cent egg suspension at 25° C. After it had been determined that the gel-dispersing agent was completely saturated by the fertilizin-gel available in a 60 per cent egg suspension, the effect of dilution of the extract was tested against 60 per cent egg suspensions.

Determination of the effect of increasing substrate concentrations

Egg suspensions were prepared ranging in concentrations from 10 to 100 per cent. The activity of two drops of undiluted partially purified extract was tested against one drop of the various egg suspensions at 25° C. The suspensions were gently agitated at 3 minute intervals to insure that all eggs were equally exposed to the dispersing agent.

Determination of pH sensitivity

The pH of crude and partially purified extracts was adjusted to various hydrogen ion concentrations, as measured by a glass electrode, by drop-wise addition of 0.1 N sodium hydroxide or 0.1 N hydrochloric acid. The dispersing activity of two drops of the extract at various

hydrogen ion concentrations was tested against one drop of 10 per cent egg suspension at 25° C. The addition of reagents to adjust the pH of the extract introduced two possible sources of error in accurately determining the activity of the gel-dispersing agent, namely, possible removal of the fertilizin-gel by acid or alkaline solution and reduction of the rate of activity due to dilution of the extract. To eliminate the effects of acid or alkaline solutions, extracts were exposed to various hydrogen ion concentrations for 1 minute and then readjusted to pH 8.0 before testing. To account for the decrease in activity due to dilution of the extract, filtered sea water was added to control extracts in volumes equal to the volume of reagent used to adjust the pH. Comparison of the activities of the preparations was then made.

Thermal stability

Crude and partially purified extracts were subjected to various temperatures, in a water bath, for 2 minutes and then cooled with tap water to 25° C. The gel-dispersing activity of two drops of the heat-treated extracts was tested against one drop of 10 per cent egg suspensions.

Interaction between the gel-dispersing agent and antifertilizin

In order to ascertain the role, if any, of antifertilizin in the dispersal of the fertilizin-gel, experiments were undertaken which were designed to eliminate antifertilizin from the sperm extracts. Tests were then made to determine the change, if any, on the rate of activity of the gel-dispersing agent.

If eggs of the sand dollar are allowed to stand in sea water for

a period of several hours, the fertilizin will diffuse from the gelatinous material into the sea water. This "egg water" will then cause the agglutination of homologous live spermatozoa. Frank (1939) has shown that when sperm extracts are added to the egg water of Arbacia punctulate, the fertilizin of the egg water is neutralized. On the assumption that the fertilizin of the egg water would combine with the antifertilizin of the sperm extracts which contained the active gel-dispersing agent and thus remove the antifertilizin, egg water with a sperm agglutination titer of 1:1000, as determined by serial two-fold dilutions of the egg water, was added to an equal portion of sperm extract and this solution was allowed to stand overnight at 1° C. A very slight precipitate was formed which was removed by centrifugation at 15,500 x gravity for 10 minutes. The supernatant was tested for dispersal activity against fresh eggs. In a control experiment, untreated extracts were diluted 1:1 with filtered sea water and tested for gel-dispersing activity. The activity rates of the two solutions were compared.

In another set of experiments, the fertilizin-gel was removed from eggs by allowing the eggs to stand in 20 milligram-per cent trypsin for 10 minutes. The denuded eggs were then washed five times in 30 volumes of sea water to remove the trypsin. Several investigators have shown that when the fertilizin-gel is thus removed, some fertilizin remains on the egg surface and this fertilizin will cause the agglutination of spermatozoa (see Metz, 1957). Two volumes of partially purified extract were then absorbed with one volume of trypsin-treated eggs for 5 minutes. The eggs were then removed by centrifugation and the absorbed extract was tested for gel-dispersing activity against fresh eggs.

The question had been raised (Brookbank, personal communication) of the possibility that antifertilizin or some other sperm component was necessary as a co-factor for the activity of the gel-dispersing agent. In this case, dilution of the sperm extract with sea water would decrease the concentration of the dispersing agent and/or the co-factor below a critical level. To explore this possibility, different aliquots of partially purified extracts were heated to 70° C., 95° C. and 100° C. respectively. The 70° C. treatment inactivated the gel-dispersing agent but did not affect the activity of the antifertilizin in the extract. Antifertilizin is stable when heated for 2 minutes at 100° C., but longer exposure to this temperature degrades it. Unheated, partially purified extracts were then diluted to 50 per cent concentrations with sea water, and with extracts which had been heated to the various temperatures. These solutions were then tested for gel-dispersal activity against fresh eggs, at 25° C.

Tests for proteins

Standard qualitative tests were made, on partially purified extracts, to determine if the active gel-dispersing agent might be protein in nature.

RESULTS AND DISCUSSION

The dispersal of the fertilizin-gel by the dispersing agent from sperm extracts appears to be the result of interaction between the active dispersing agent and the surface molecules of the fertilizin-gel. As shown in Figure 1, the ring of echinochrome granules lies in the outer one-third of the layer of fertilizin-gel. It was noted that, in every instance, the time required for the dispersal of the gel outside the ring of echinochrome granules was much greater than that required for the dispersal of the remainder of the fertilizin-gel, residing between the granules and the egg surface. For example, under conditions in which the dispersal of the echinochrome granules was apparent in 6 minutes, the eggs were completely denuded in about 10 minutes. When all of the fertilizin-gel has been removed, the surfaces of the eggs may lie in direct contact. Thus, complete removal of the gel can be detected without the aid of stains. If the volume of fertilizin-gel outside the ring of echinochrome granules is compared with that residing between the granules and the egg surface, one finds that the mean volume outside the granules is 7.0×10^{-4} cubic millimeters per egg while the volume inside the granules is 2.5×10^{-3} cubic millimeters. It is assumed that the quality of the fertilizin-gel is homogeneous throughout the thickness of the gel surrounding the eggs. The homogeneity of the fertilizin-gel would, it appears, depend upon the cellular origin of the gel. The question of this origin has not

been clarified (see Metz, 1957). If the rate of dispersal activity were dependent upon the volume of gel present, the inner portion of the gel would be dispersed more slowly. Actual measurements of the change in the diameter of the fertilizin-gel with time, after the gel had been exposed to the dispersing agent, were attempted. However, the stain used to outline the fertilizin-gel adversely affected gel dispersal and attempts at measurement were unsuccessful. If one considers that the surface is decreasing at a rate which is less than the rate of volume decrease, one would expect a more rapid dispersal of the inner portion of the fertilizin-gel, provided one is dealing primarily with a surface phenomenon, and with a homogeneously dense gelatinous coat around the egg. Consideration of surface-volume relationships would be of prime importance if one were concerned with absolute rate of dispersal of the fertilizin-gel. In this study, time of dispersal was recorded only to the point at which the echinochrome granules began to fall to the bottom of the Syracuse dish and change in rate of dispersal was not employed and calculations were based on the volume of fertilizin-gel dispersed. In future studies, during which more refined measurements will be made, the change in rate must be considered.

Rates of gel-dispersal activity

The results of experiments to determine the rates of dispersal activity at various extract concentrations are summarized in Figure 3. Since the substrate concentrations were held constant in these experiments, the rate of gel dispersal was assumed to be independent of the substrate concentration and to be a function of the concentration of the active dispersing agent. The curve in Figure 3 is

similar to the curve obtained when a zero-order enzymatic reaction is plotted (Neilands and Stumpf, 1955). However, it was not known whether an excess substrate was present in these experiments. Subsequently, experiments were performed in which various concentrations of extracts were tested against 60 per cent egg suspensions, conditions under which the substrate was known to be in excess. The results of these experiments, when plotted (Figure 4), produce a curve similar to that in Figure 3, except that the critical level of dilution of the extract is higher when the substrate concentration is increased. In the earlier experiments the substrate may have been in excess relative to the dilute extract concentrations. It is also possible that there were variations in the potency of the extract preparations from different spawning seasons that gave a misleading picture of the dispersal rates.

The results of experiments in which the concentration of the gel-dispersing agent was kept constant and the substrate concentration was varied are described in Figure 5. It will be noted that with lower substrate concentrations a zero-order plot is described, but at higher substrate concentrations the curve more or less describes a first-order reaction. It is suggested that under the conditions present in these experiments, the gel-dispersal reaction is one which can not be described by a single plot. One must bear in mind that the gel dispersal observed is due to an agent in impure form. Studies are projected in which it is hoped that the kinetics of a relatively pure gel-dispersal enzyme may be described. There is an inherent difficulty present in attempting to record the time of dispersal at high concentrations of eggs. In egg suspensions above 60 per cent, the eggs are so dense that

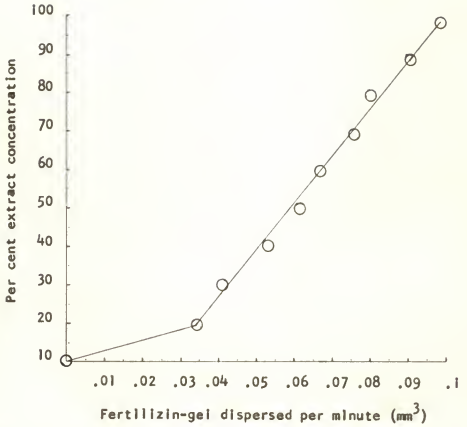

Figure 3. The effect of dilution of sperm extracts on the rate of fertilizin-gel dispersal when the dispersing agent is not saturated.

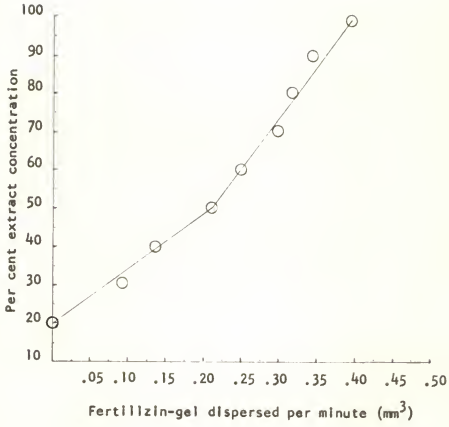

Figure 4. The effect of dilution of sperm extracts on the rate of gel dispersal when substrate is in excess.

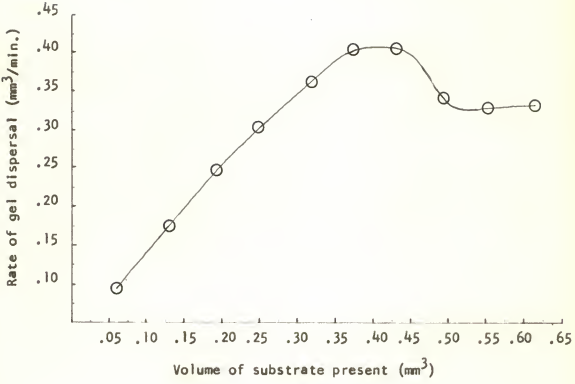

Figure 5. Rate of dispersal of fertilizin-gel with varied substrate concentrations.

it is difficult to accurately determine whether granules are being dispersed, and thus record the end-point in the measurements. Although efforts were made to insure that all eggs were equally exposed to the dispersing agent, it is possible that in dense egg suspensions all eggs were not equally exposed. Therefore, what appears in Figure 5 to be a decrease in activity at high substrate concentrations could actually be an expression of error in determining the time at which the echinochrome granules were dispersed and/or inadequate exposure of the eggs to the dispersing agent. One would like to be able to determine the amount of product formed in these reactions, but no satisfactory method of separating the product from the solution has, as yet, been devised.

pH sensitivity

The optimum pH for dispersal of the fertilizin-gel of sand dollar eggs by homologous sperm extracts was shown by Brookbank (1958) to be 8.0. The present investigation confirms this observation. When gel dispersal was attempted at higher pH values there was a gradual decrease in activity up to pH 9.8 at which point no dispersal was apparent (Table 1). When sperm extract was exposed to pH 9.8 and readjusted to pH 8.0 the extract was incapable of causing the dispersal of the fertilizin-gel (Table 2). It is apparent that the active dispersing agent was denatured at pH 9.8. Acidification of sperm extracts also resulted in decreased dispersal activity. Extracts exposed to pH 4.0 for 1 minute and then readjusted to pH 8.0 were incapable of causing the dispersal of the fertilizin-gel (Table 2). The activity of the antifertilizin present in the sperm extracts was not altered by this acid treatment. The data in Table 1 and Table 2 are those obtained from partially purified

Table 1. Effects of pH on the gel-dispersing capacity of sperm extracts.

Tested at pH	Time, in minutes, required for gel dispersal	
	Treated extract	Control
6.2	50.0	21.0
6.4	40.0	19.0
6.6	31.0	17.5
6.8	29.0	16.5
7.0	26.0	16.0
7.2	25.0	15.0
7.4	10.0	9.0
7.6	9.5	8.0
7.8	8.5	8.0
8.0	7.5	7.5
8.2	8.0	8.0
8.4	9.5	8.5
8.6	10.0	9.0
8.8	11.0	9.5
9.0	11.5	9.5
9.2	13.5	11.0
9.4	17.0	12.0
9.6	19.0	13.0
9.8	-	15.0

The mean values from five experiments are given. Minus signs indicate no dispersal activity after one hour.

Table 2. Effects of exposure of sperm extracts to various hydrogen ion concentrations.

Extract exposed to pH	Time, in minutes, required for gel dispersal
10.0	-
9.8	-
9.5	13.0
9.0	9.5
8.5	7.0
8.0	6.0
7.5	6.5
7.0	8.5
6.5	9.5
6.0	10.0
5.5	15.0
5.0	20.0
4.5	25.0
4.0	-

Times shown are the average dispersal times obtained from four experiments, using different extract preparations. Minus sign indicates no gel-dispersal activity in one hour.

extracts. No apparent difference in the effects of changes in pH on crude and partially purified extracts was observed.

Thermal stability

The effects of temperature on the stability of both crude and partially purified extracts are quite clear, as shown in Table 3. This table summarizes the results of experiments involving five different preparations of extract. It is apparent from these data that partial purification of sperm extracts enhanced their gel-dispersing capacity and also enhanced the thermal stability, up to a temperature of 45° C., at which point the gel-dispersing agents in all extracts were inactivated. This heat treatment did not inactivate the antifertilizin present in the extract.

It had been the observation of the present investigator that partially purified extracts remained active over a longer period of time in storage than did the crude extracts. The experiments on thermal stability provided some degree of confirmation for this observation. In these experiments tests were made on extracts within three days of their preparation, with one exception. The tests conducted on January 9, 1961, were made with extract prepared on December 14, 1960. It will be noted that the crude extract from this preparation was less active than the extracts from the other preparations.

Interaction between the gel-dispersing agent and antifertilizin

When sperm extracts were absorbed with egg water, no decrease in gel-dispersing activity was apparent that could not be attributed to dilution of the dispersing agent. As shown in Table 4, the dispersal times for absorbed extract, at various dilutions, were essentially the

Table 3. The effect of temperature on the gel-dispersing activity of crude and partially purified sperm extract.

Date	Temperature in degrees Centigrade				
	25°	30°	35°	40°	45°
12/30/60	9.5	10.5	13.5	30.0	no
	7.5	9.5	11.5	22.0	dispersal
1/9/61	15.0	22.0	29.5	36.0	"
	10.0	10.5	12.0	16.0	"
1/10/61	16.0	17.5	19.5	24.5	"
	13.0	14.0	15.5	21.0	"
5/14/61	7.0	8.5	12.0	16.0	"
	6.0	7.5	9.0	13.5	"
5/31/61	7.5	9.0	11.5	15.5	"
	6.0	7.0	9.5	13.0	"

The figures in the columns represent the time, in minutes, required for dispersal of the fertilizin-gel after the extract had been exposed to the corresponding temperature for 2 minutes. The time required for dispersal by crude extract is recorded in the upper line opposite each date.

same as for normal extracts diluted with sea water. While no claims are made which would imply that all the antifertilizin had been absorbed by the egg water, it is certain that a portion of the antifertilizin had been removed. The loss of a portion of the antifertilizin would have resulted in decreased gel-dispersal activity, if the dispersal had been a function of the antifertilizin. In these experiments the decrease in gel-dispersal activity was presumably due to dilution of the active gel-dispersing agent and not to the loss of antifertilizin.

The results of the experiments in which partially purified extracts were diluted with extracts which had been subjected to various temperatures are summarized in Table 5. Antifertilizin in sperm extracts can be detected by the presence of a precipitation membrane formed on the surface of the fertilizin-gel when fresh eggs are exposed to the extracts. In the presence of the fertilizin-gel dispersing agent, only a very faint precipitation membrane could be seen. When the gel-dispersing agent was inactivated by heating, a precipitation membrane was more readily formed by the antifertilizin remaining in the extract. When sperm extracts were boiled for 5 minutes, then tested against fresh eggs, the formation of a precipitation membrane could not be detected. This indicated that some degree of inactivation of the antifertilizin had occurred. The data in Table 5 indicate that some substance was present in the heated extracts that enhanced the gel-dispersing capacity of the unheated, partially purified extracts. The presence of some such substance is also suggested by the curves in Figures 3 and 4 which show a critical dilution point. One might expect this to occur if more than one agent was involved.

Absorption of sperm extracts with trypsin-treated eggs produced

Table 4. Effects of absorption of extracts with fertilizin on the gel-dispersing capacity of the extracts.

Date	Dilution extract/egg Water	Time, in minutes, required for gel-dispersal	
		Absorbed extract	control
12/11/60	5/0	9.0	9.0
	5/1	10.5	10.5
	5/2	11.0	11.0
	5/3	12.5	11.5
	5/4	13.0	13.0
	5/5	16.0	15.0
12/11/60	5/0	7.0	7.0
	5/1	8.0	8.0
	5/2	11.0	11.0
	5/3	12.5	12.5
	5/4	13.5	13.5
	5/5	15.5	15.0
12/16/60	5/0	9.0	9.0
	5/1	10.0	10.5
	5/2	11.0	11.0
	5/3	13.0	13.0
	5/4	14.5	14.5
	5/5	16.0	16.0
1/10/61	5/0	10.0	10.0
	5/1	11.0	11.0
	5/2	12.0	12.5
	5/3	13.5	13.5
	5/4	14.5	14.5
	5/5	16.0	15.5

Table 5. Effects of dilution of sperm extracts with heat-treated extracts and with sea water.

Treatment of active extracts	Time, in minutes, required for gel dispersal in a 10% egg suspension			
Undiluted	6.0	6.0	6.5	6.5
Diluted with 70° C. - treated extract	8.0	8.0	9.0	10.5
Diluted with 95° C. - treated extract	8.0	8.0	9.0	10.5
Diluted with 100° C. - treated extract	8.0	8.0	9.0	10.5
Diluted with sea water	10.0	10.0	11.5	13.5

The columns represent four different sets of experiments.

profound effects on the dispersing agent. Extracts which caused the dispersal of fertilizin-gel in 6 minutes required 45 - 55 minutes to disperse an equal volume of fertilizin-gel after absorption against trypsin-treated eggs. This phenomenon was, at first, thought to be caused by inadequate washing of the eggs to remove the trypsin. To determine if this was the case, fertilizin-gel was removed by subjecting eggs to pH 3.5 sea water for 1 minute. The acid sea water was quickly decanted and the eggs were washed three times in 30 volumes of normal sea water. By this procedure all traces of acidity were eliminated from the eggs. Sperm extracts were then absorbed against the acid-denuded eggs according to the procedure outlined for trypsin-treated eggs. When the absorbed extract was recovered, it was found to be incapable of causing the dispersal of the fertilizin-gel in less than 45 minutes.

Protein nature of the fertilizin-gel dispersing agent

Positive results were obtained from biuret, ninhydrin and xantho-proteic tests on partially purified extracts. These tests, in themselves, do not constitute proof of the protein nature of the active gel-dispersing agent. The agent is non-dialyzable, precipitated with ammonium sulphate, heat-labile and is relatively sensitive to changes in pH. These characteristics, together with the positive results of qualitative tests causes the present author to conclude that the fertilizin-gel dispersing agent is a protein. There is also evidence that the active substance depolymerizes the fertilizin-gel in a manner characteristic of enzyme activity.

Role of the gel-dispersing enzyme in the fertilization of eggs

While most of the experimental results recorded here are straightforward, some of the results project implications of a theoretical nature. Brookbank (1958) demonstrated that if a fertilizin solution was mixed with an equal volume of sperm extract, the sperm agglutination titer of the fertilizin was not decreased. Because of this, he suggested that the gel-dispersing agent depolymerized the fertilizin-gel, leaving the fertilizin molecule essentially unaltered. Tyler (1939) concluded that the membrane lysin obtained from the spermatozoa of Megathura crenulata functioned in enabling the spermatozoa to pass through the egg membranes. Under natural conditions of fertilization, the egg membranes or fertilizin-gel are not completely dispersed. It is believed that the individual spermatozoan possesses a sufficient quantity of the gel-dispersing agent to facilitate passage through the barriers surrounding the egg. Colwin and Colwin (1960), by means of electron microscopy, have shown that a single spermatozoan of Hydroides hexagonus could cause the formation of a sperm entry hole through the vitelline membrane of homologous eggs. With regard to the fertilizin-gel dispersal in M. quinquelesperforata, there is no reason to postulate a function other than that of allowing the passage of spermatozoa through the fertilizin-gel.

There is a reaction between the gel-dispersing agent and the vitelline membrane, in M. quinquelesperforata, that has not been made clear by this study. If eggs were fertilized and then exposed to the sperm extracts, crenation of the fertilization membrane was observed. When sperm extracts were absorbed with denuded eggs, not only were the extracts rendered practically inactive, but the surfaces of the eggs were

also altered, leaving the surfaces in a crenated condition. It will be recalled that denuded eggs exposed to sperm extracts are incapable of fertilization by live sperm. It is possible that the sperm-receptor sites on the egg surface are neutralized by the activity of the sperm extract. Brookbank (1958) showed that the gel-dispersing enzyme in M. quinquiesperforata caused the dissolution of precipitation membranes that were formed on the fertilizin-gel prior to the addition of the enzyme. Rothschild (1956) pointed out that the reversal of agglutinated spermatozoa might be brought about by the mechanical action of the live spermatozoa or by the activity of chemical agents. Observations made during this study provide evidence that the gel-dispersing enzyme is not responsible for the reversal of agglutination in M. quinquiesperforata. Live spermatozoa, which possess the enzyme, can be agglutinated by fertilizin. Reversal of agglutination will then occur and the reversed spermatozoa are incapable of re-agglutination. If the gel-dispersing enzyme caused the reversal, it would appear that the spermatozoa would be capable of re-agglutination. The fertilizin-antifertilizin reaction is not reversible in vitro, although the enzyme is present in sperm extracts. The apparent dissolution of the precipitation membranes may be caused by depolymerization of the fertilizin-gel underlying the membranes, which could cause the membrane to break up into small, possibly soluble, fertilizin-antifertilizin complexes.

It is of considerable interest that fertilizin-reversed spermatozoa of M. quinquiesperforata and A. punctulata are capable of fertilizing fresh eggs (Tyler and Metz, 1955). Both species possess

gel-dispersing enzymes. The depolymerization of the fertilizin-gel by the gel-dispersing enzyme would enhance the "muzzling" of the spermatozoa by fertilizin as the spermatozoa pass through the gel. This "muzzling" of the spermatozoa in the two species mentioned is, apparently, of no serious consequence since fertilization of the egg by fertilizin-reversed sperm can occur. The significance of the ability of reversed spermatozoa to fertilize eggs remains sufficiently obscure to merit further study.

SUMMARY AND CONCLUSIONS

A fertilizin-gel dispersing agent from the spermatozoa of the sand dollar, Mellita quinquiesperforata, has been studied. It has been demonstrated that the active dispersing agent is not antifertilizin. The agent is believed to be a protein and its activity suggests that it is an enzyme. The gel-dispersing agent is non-dialyzable, precipitated by ammonium sulphate and gives positive biuret, ninhydrin and xanthoproteic reactions. The enzyme is denatured by exposure to 45° C. for 2 minutes. Optimum pH for gel-dispersal is 8.0. Exposure to either pH 4.0 or 9.8 for 1 minute inactivates the enzyme. Gel-dispersal capacity of the enzyme is greatly reduced when the enzyme is absorbed with denuded eggs. Evidence is given that a heat-stable cofactor from the spermatozoa is necessary for the optimal activity of the enzyme.

The gel-dispersing enzyme apparently depolymerizes the fertilizin-gel surrounding homologous eggs and probably functions in nature to facilitate the passage of the spermatozoa through the fertilizin-gel. Reversal of fertilizin-agglutination of spermatozoa is evidently not caused by the gel-dispersing enzyme. The "muzzling" of spermatozoa by depolymerized fertilizin is believed to be compensated for by the ability of fertilizin-reversed spermatozoa to fertilize homologous eggs.

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BIOGRAPHICAL SKETCH

John Marvin Rawls was born November 25, 1920, near Springfield, Tennessee. In June, 1939, he graduated from Bell High School, Adams, Tennessee. From 1942 to 1945 he served in the United States Army Air Force as a pilot and saw combat duty in Europe. In 1949 Mr. Rawls entered George Peabody College for Teachers and received the Bachelor of Science and Master of Arts degrees in 1953. From 1953 to 1957 he taught biology at Austin Peay State College, Clarksville, Tennessee. In 1957 he enrolled in the Graduate School of the University of Florida to pursue work toward the degree of Doctor of Philosophy. During his tenure at the University of Florida he has held National Science Foundation Faculty Science Fellowships, a Southern Fellowship and has served as a teaching assistant.

John Marvin Rawls is married to the former Edna Ruth Schleicher and is the father of three children. He is a member of the American Society of Zoologists, the Association of Southeastern Biologists, Tennessee Academy of Science, Phi Sigma Society, Society of Sigma Xi and Kappa Phi Kappa.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 12, 1961

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